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Inhibition of the mammalian target of rapamycin complex 1 signaling pathway reduces itch behaviour in mice

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Abstract

Activated mammalian target of rapamycin (P-mTOR) has been shown to maintain the sensitivity of subsets of small-diameter primary afferent A-nociceptors. Local or systemic inhibition of the mTOR complex 1 (mTORC1) pathway reduced punctate mechanical and cold sensitivity in neuropathic pain and therefore offered a new approach to chronic pain control. In this study, we have investigated the effects of the rapamycin analog temsirolimus (CCI-779) on itch. Bouts of scratching induced by the histamine-dependent pruritogenic compound 48/80 and histamine-independent pruritogens, chloroquine and SLIGRL-NH₂, injected intradermally were significantly reduced by local (intradermal) or systemic (intraperitoneal, i.p.) pretreatment with CCI-779. We also investigated the action of metformin, a drug taken to control type 2 diabetes and recently shown to inhibit mTORC1 in vivo. Although the response to nonhistaminergic stimuli was reduced at all of the time points tested, scratching to compound 48/80 was modified by metformin only when the drug was injected 24 hours before this pruritogen. We also examined the colocalization of P-mTOR with gastrin-releasing peptide, a putative marker for some itch-sensitive primary afferents, and found that P-mTOR was coexpressed in less than 5% of gastrin-releasing peptide-positive fibers in the mouse skin. Taken together, the data highlight the role that P-mTOR-positive A-fibers play in itch signaling and underline the importance of the mTORC1 pathway in the regulation of homeostatic primary afferent functions such as pain and itch. The actions of the antidiabetic drug metformin in ameliorating nonhistamine-mediated itch also suggest a new therapeutic route for the control of this category of pruritus.

Keywords: Mammalian target of rapamycin complex 1, Gastrin-releasing peptide, Temsirolimus (CCI-779), Metformin, Itch, Mice

1. Introduction

Small-diameter primary afferent fibers have been classified in a number of ways principally according to their receptive field properties, complement of receptor proteins, and neurotransmitter or protein kinase content.^{10,15,30,44} In some cases, it has been possible to demonstrate that particular molecular signatures correspond to functional subtypes of primary afferent. For example, nociceptors that respond to chloroquine and signal itch, but not pain, can be identified by the expression of specific members of the Mas family of G-protein-coupled receptors (MrgprA3).¹² Also, gastrin-releasing peptide (GRP)-expressing

primary afferents have also been proposed to play a role in itch sensation.^{42,45}

Our previous work identified 1 group of small-diameter A δ -nociceptors as containing an activated form of the mammalian target of rapamycin (mTOR), a kinase that maintains the excitability of these A-fibers.^{9,18,31} mTOR belongs to the phosphatidylinositol 3-kinase-related kinase protein family and forms at least 2 multiprotein complexes known as mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2).²⁴ Although mTORC1 is well understood and recognised as an evolutionarily conserved environmental sensor signaling changes to diverse cues and displays acute sensitivity to rapamycin,⁴⁷ much less is known about mTORC2. In a phosphorylated form, mTORC1 (P-mTORC1) can activate downstream pathways involving ribosomal protein S6K (S6 kinase) and 4EBP (eIF4E; eukaryotic initiation factor 4E-binding protein) and has been shown to promote protein synthesis in cell bodies, axons, and dendrites.^{7,19,32,47} This kinase also can be negatively regulated by the adenosine monophosphate-activated protein kinase (AMPK) through the activation of mTORC1's negative regulator tuberous sclerosis complex 2.^{6,8} This results in a profound inhibition of mTORC1 and its downstream targets involved in translational control (eg, 4EBP and S6 kinase).^{6,8} Our previous investigations revealed that a subset of P-mTORC1 containing A-nociceptive primary afferents supported the increased punctate mechanical sensitivity that develops around a site of injury (also referred to as secondary hyperalgesia) but did not influence peripheral sensitization or acute pain responses.^{9,18,31} For example, it was shown that blocking the actions of mTORC1

Sponsorships or competing interests that may be relevant to content are disclosed at the end of this article.

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peripherally and/or systemically with inhibitors such as rapamycin, the rapamycin ester temsirolimus (CCI-779), or an ATP-competitive inhibitor Torin-1 reduced both the spread of punctate mechanical sensitivity after inflammation or local injury and mechanical hypersensitivity in animal models of neuropathic pain. Importantly, mTORC1 inhibitors did not modulate the C-fiber-evoked peripheral or central sensitization induced by capsaicin injection, confirming A-fibers as the principle target for locally and/or systemically applied mTORC1 inhibitors.^{30,31,33}

Here, we have extended the list of functions mediated by mTOR-positive primary afferents and investigated the involvement of mTORC1 in itch signaling. Although recent research has emphasized the role of C-fibers in pruritus research, there is evidence that some A-fibers are also sensitive to the 2 classes of histaminergic and nonhistaminergic stimuli widely used to induce itch.^{4,11,27,28,34} Thus, we investigated the antipruritic action of CCI-779 and metformin, a drug widely given to treat type 2 diabetes and recently shown to inhibit mTORC1 signaling through AMPK pathway found in many cell types including neurons.^{6,8,26}

2. Materials and methods

2.1. C57BL/6J mice

Adult male C57BL/6J (B6) mice (8 weeks of age; 25–30 g; Harlan OLAC, Bicester, United Kingdom) were allowed to acclimate to the colony room (Central Biological Services; University College London, United Kingdom) for at least 7 days after arrival and were housed in polyethylene cages (4 per cage), controlled for temperature (21°C) and humidity (55%) under a regular 12-hour day/night cycle (lights on at 8:00 AM and lights off at 8:00 PM). Standard laboratory rodent chow and water were available *ad libitum*. All efforts were made to minimize animal suffering and to reduce the number of animals used in the study. Experimental protocols were approved by the Institutional Animal Care and Use Committee of our institution and were consistent with the guidelines provided by the UK Animals (Scientific Procedures) Act 1986 and the guidelines of the Committee for Research and Ethical Issues of IASP published in PAIN, 16 (1983): 109–10.

2.2. Preparation and administration of drugs

2.2.1. CCI-779

For intradermal (i.d.) administration, CCI-779 (temsirolimus; Cat. No. T-8040; LC Laboratories, Woburn, MA, USA) at a dose of 12.5 nmol (ie, 50 µL of 250 µM solution) was prepared immediately before injections in a vehicle solution containing 0.15 M NaCl, 5% polyethylene glycol 400, 5% Tween 20, and 20% ethanol, similarly to our previous study.³¹ Intradermal injections were given over 1 minute in a volume of 50 µL in anaesthetized mice. Control animals received 50 µL of equivalent vehicle solution without CCI-779. For systemic (intraperitoneal [i.p.]) administration, CCI-779 was prepared in pure ethanol as a stock solution at 60 mg/mL and diluted to 2.5 mg/mL in 0.15 M NaCl, 5% polyethylene glycol 400, 5% Tween 20 immediately before injections, similarly to our previous study.³¹ Mice were weighed and then injected i.p. with a 1% vol/wt solution of CCI-779 (25 mg per kg body weight) or an equivalent vehicle solution without CCI-779 as a control group. CCI-779/vehicle was administered once 6 hours before injection of pruritogens. The concentration and

timing of CCI-779 injections were based on our previously published report.³¹

2.2.2. Metformin

For systemic (i.p.) administration, metformin (metformin hydrochloride; Cat. No. 2864; Tocris Bioscience, Bristol, United Kingdom) was prepared in sterile saline immediately before injections. Mice were weighed and then injected i.p. with metformin (200 mg per kg body weight) or equivalent vehicle solution without metformin as a control group. Metformin/vehicle was administered once 4 or 24 hours before injection of pruritogens. The concentration and timing of metformin injections were based on both previously published research using CCI-779, rapamycin, or metformin^{18,26,31} and on our own pilot data in rats suggesting that both CCI-779 and metformin were effective in increasing thermal activation thresholds maximally in A-fibers 3 to 4 hours after systemic i.p. treatment. Indeed, mTORC1 signaling was inhibited by metformin in dissociated mouse trigeminal neuron cultures within 1 hour.²⁶

2.3. Induction of scratching behaviour

As previously published,^{42,43} itch was induced in B6 mice by injection of pruritogens: histamine-dependent compound 48/80 (100 µg; Cat. No. C2313; Sigma-Aldrich, Suffolk, United Kingdom) and histamine-independent chloroquine (chloroquine diphosphate salt, 200 µg; Cat. No. C6628; Sigma-Aldrich) or peptide SLIGRL-NH2 (100 µg; Cat. No. H-5078; Bachem, Bubendorf, Switzerland; SLIGRL-NH2 was thought to be a PAR-2 agonist, but it has been shown that SLIGRL-NH2 injection generates a histamine-independent itch through the MrgprC11 receptor while a PAR2 ligand caused thermal hypersensitivity²²). Itch-inducing agents were dissolved in sterile saline and immediately administered by i.d. injection into the nape of the mouse neck in a volume of 50 µL. Before itch experiments, the back of the mouse neck was shaved and animals were given 30 minutes to acclimate to a small plastic chamber. For i.d. injections of pruritogens, mice were removed from the chamber, gently restrained, and i.d. injected. Itch behaviour in the mouse that developed after i.d. injection of pruritogens was recorded with a digital video camera and analysed. One scratch was defined as a lifting of the hind limb toward the injection site (the shaved area of the neck) and then replacing the limb back to the floor, regardless of how many scratching strokes (bouts of scratching) took place between those 2 movements.³⁸ The scratching was quantified as the total number of scratches across 40-minute observation period plus the cumulative number of scratches observed for 40 minutes at 5-minute intervals. In all experiments, the observer was not aware of the substance and/or dose given. Each mouse was used only once, in 1 experiment.

2.4. Biochemical assays

2.4.1. Immunohistochemistry

To localize P-mTOR and GRP, naive mice were deeply anaesthetized with pentobarbital and perfused transcardially with saline containing 5000 IU/mL heparin followed by 4% paraformaldehyde in 0.1 M phosphate buffer containing 0.05 M sodium fluoride (~100 mL per mouse). The glabrous skin of the hind paw around the foot pads and the hairy skin from the back of the neck were dissected out, postfixed in the same paraformaldehyde solution for 2 hours at 4°C and transferred into a 30% sucrose solution in

phosphate buffer containing 0.01% azide for a minimum of 24 hours. Tissue sections were cut at 40 μm on a freezing microtome perpendicular to the surface of the skin.

Sections of the skin were blocked in PBS containing 0.2% Triton X-100 and 5% normal goat serum for 1 hour at room temperature. Sections were then left to incubate with rabbit anti-phospho-mTOR (P-mTOR, Ser2448; 1:1000; Cat. No. 2971; Cell Signaling Technology Leiden, The Netherlands) for 3 days at 4°C. A tyramide signaling amplification-based protocol was used to amplify the P-mTOR signal.^{9,31} Appropriate biotinylated secondary antibodies were used at a concentration of 1:400 for 90 minutes. Samples were then incubated with avidin-biotin complex (ABC Elite; Vector Laboratories, Peterborough, United Kingdom) (1:250 Vectastain A + 1:250 Vectastain B) for 30 minutes followed by a signal amplification step with biotinylated tyramide solution (1:75 for 7 minutes; Perkin Elmer Llantrisant, United Kingdom). Finally, sections were incubated with FITC-avidin for 2 hours (1:600; Vector Laboratories). The sections were then reprobed with rabbit anti-GRP (1:100; Cat. No. 20073; Immunostar, Newmarket, United Kingdom) for 24 hours at room temperature to determine cellular colocalization. The appropriate directly labeled secondary antibody was applied at a concentration of 1:500 and incubated for 2 hours. All sections were coverslipped with Gel Mount Aqueous Mounting Medium (Sigma-Aldrich) to protect the fluorescence from fading and stored in dark boxes at 4°C. Controls included omission of the first or second primary antibodies. All images of double-stained tissue were acquired by confocal microscopy using a laser scanning microscope (Leica TCS NT SP) as described previously.^{9,31} Counts were taken from confocal images taken

using a 40 \times objective. Sections at least 240 μm apart taken from each of the 3 mice were sampled, and a total of 30 to 50 GRP-positive fibers were analysed for P-mTOR-positive immunoreactivity. Postacquisition processing was performed with Adobe Photoshop and Adobe Illustrator.

2.4.2. Immunoblotting

CCI-779 inhibition of mTORC1 activity was shown previously.^{9,18,31} To verify the efficacy and specificity of mTORC1 inhibition by metformin in our experimental setting, we evaluated activation of downstream components of the mTORC1 signaling pathway in treated mice. Ribosomal protein S6K (S6 kinase) and 4EBP (eIF4E; eukaryotic initiation factor 4E-binding protein) are well-characterized substrates for mTORC1. S6K activation absolutely requires TORC1-mediated phosphorylation and phosphorylates its own set of targets, many of which promote protein production.²⁴ In a parallel pathway, TORC1-mediated phosphorylation of 4EBP1 initiates cap-dependent translation by eIF4E.²⁴ Therefore, we evaluated levels of phosphorylation of mTOR (P-mTOR/mTOR), S6 ribosomal protein (P-S6/S6), and 4E-BP1/2 (P-4E-BP1/2/4E-BP1/2) as indicators of inhibition of the mTORC1 pathway.

Mice were killed with CO₂, decapitated, and the lumbar (L4-L6) dorsal spinal cord and dorsal roots from naive animals 4 and 24 hours after i.p. injection of metformin and its appropriate vehicle controls were quickly dissected out and frozen on dry ice. Samples were then stored at -80°C until further processing. For protein extraction, samples were homogenized by sonication in lysis buffer (1% Np-40, 20 mM Hepes pH 7.4, 100 mM NaCl, 100 mM NaF, 1 mM

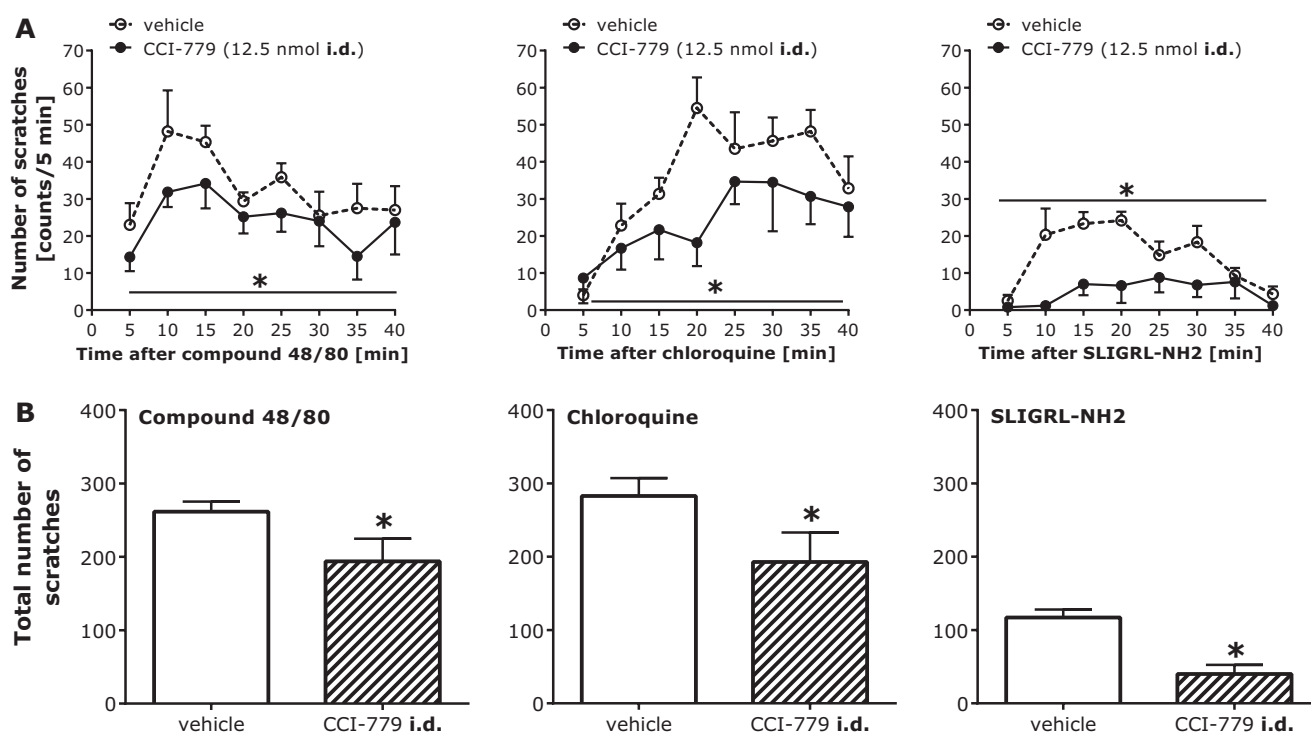


Figure 1. Intradermal (i.d.) administration of CCI-779 inhibited itch behaviour induced by histamine-dependent and histamine-independent pruritogens in mice. (A) Time-course effect of i.d. injection of CCI-779 (12.5 nmol), or vehicle, on the total number of scratches that occurred over a 40-minute time period after i.d. injection of compound 48/80, chloroquine, or SLIGRL-NH2 into the nape of the mouse neck. Mice received an i.d. injection of CCI-779 or vehicle 6 hours before the injection of pruritic agents. Itch behaviour was recorded and scratches were counted in 5-minute intervals for 40 minutes. (B) Bar graphs displaying a total number of scratches across a 40-minute observation period for each treatment. Data are presented as mean \pm SEM values, $n = 5$ to 6 in each group. The asterisk (*) denotes significance vs vehicle control animals; * $P < 0.05$ (A, 2-way analysis of variance, followed by Bonferroni comparison post hoc test; B, unpaired student t -test).

Na_3VO_4 , 5 mM EDTA with $1\times$ protease inhibitor cocktail [Sigma]; $1\times$ phosphatase inhibitor cocktail I and II [Sigma-Aldrich]) and incubated on ice for 2 hours. Samples were then centrifuged at 12,000 rpm for 15 minutes and supernatants were collected. Total protein concentration was assessed using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA) before each preparation of protein samples. Tissue extracts (15–30 μg of proteins per well) were denatured and run on 12% Bis-Tris gels (Biorad, Hemel Hempstead, United Kingdom). Proteins were electrophoretically transferred onto a PVDF membrane (Biorad Hemel Hempstead, United Kingdom). After blocking nonspecific binding site in 10 mM Tris-HCl pH = 7.5, 150 mM NaCl, 0.05% Tween 20 (Sigma) with 0.24% I-Block (Applied Biosystems, Paisley, United Kingdom) for 1 hour at room temperature, membranes were incubated overnight at 4°C with primary antibodies as listed below. After three 10-minute washes, an appropriate horseradish peroxidase (HRP)-conjugated secondary antibody was applied for 1 hour at room temperature. HRP activity was visualized by applying a chemiluminescent substrate (ECL; Amersham Pharmacia Biotech, Little Chalfont, United Kingdom) or Pierce SuperSignal West Femto (Thermo Fisher Scientific, Rockford, IL, USA) and using ChemiDoc XRS (Biorad Hemel Hempstead, United Kingdom). Membranes were then washed and incubated with GAPDH antibody overnight at 4°C and further processed as described above. Primary antibodies were detected against phosphorylated rabbit mTOR (P-mTOR, Ser2448; 1:500; Cat. No. 2971), rabbit S6 ribosomal protein (P-S6, Ser235/236; 1:200; Cat. No. 2211), rabbit 4E-BP1/2 (P-4E-BP1/2, Thr37/46; 1:200; Cat. No. 9495), and total rabbit mTOR (mTOR; 1:500; Cat. No.

2972), rabbit S6 (S6; 1:200; Cat. No. 2217), rabbit 4E-BP1/2 (4E-BP1/2; 1:200; Cat. No. 9452) (Cell Signaling Technology, Leiden, The Netherlands), as well as against mouse GAPDH (1:000; Millipore Bioscience Research Reagents, Watford, United Kingdom) used as loading control.

Immunoreactive levels for all proteins were quantified by integrating band density X area using computer-assisted densitometry (Image J; NIH, MD). The density X area measurements were normalized to that of its appropriate GAPDH signal to provide a protein/GAPDH ratio and expressed as a percent change from the mean value of controls treated with vehicle for each individual protein. The optical density of the phosphoprotein bands was divided by that of the corresponding total protein band to yield a phosphoprotein/total protein ratio. For each condition, a minimum of 3 to 6 replicates from 3 to 6 different animals were run. For an extra confirmation of equal loading of proteins, membranes were stained with Ponceau dye and staining of the wells visually compared.

2.5. Statistical data analysis

Data analysis and statistical comparisons were performed using GraphPadPrism, version 6.00 for Windows (GraphPad Software, CA, www.graphpad.com).

Behavioural and immunoblotting results are presented in the graphs as mean \pm SEM. Each group included 5 to 6 animals in behavioural experiments or 3 to 6 animals in immunoblotting approaches. Statistical analysis was performed by 2-way analysis of variance with Bonferroni multiple comparison post hoc tests for behavioural results and by

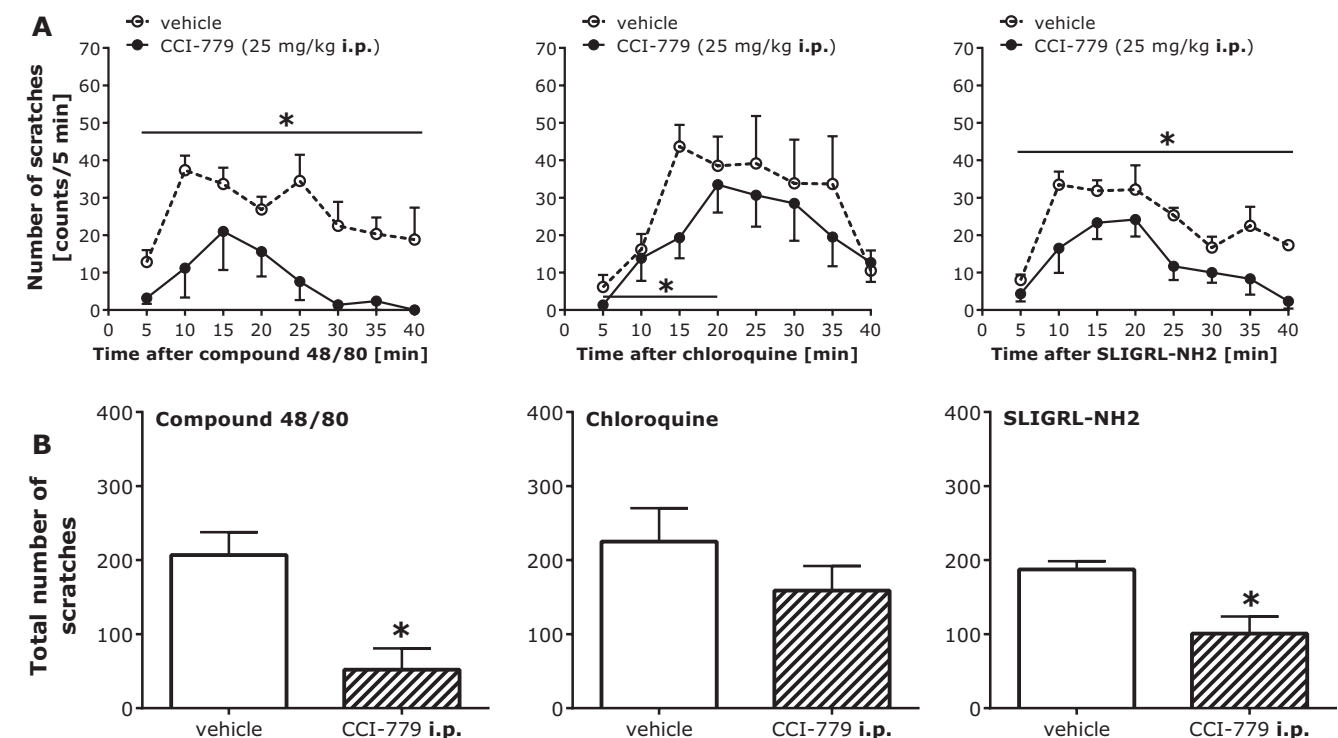


Figure 2. Intrapерitoneal (i.p.) administration of CCI-779 inhibited itch behaviour induced by histamine-dependent and histamine-independent pruritogens in mice. (A) Time-course effect of i.p. injection of CCI-779 (25 mg/kg), or vehicle, on the total number of scratches that occurred over a 40-minute time period after i.d. injection of compound 48/80, chloroquine, or SLIGRL-NH2 into the nape of the mouse neck. Mice received an i.p. injection of CCI-779 or vehicle 6 hours before the injection of pruritic agents. Itch behaviour was recorded and scratches were counted in 5-minute intervals for 40 minutes. B, Bar graphs are a total number of scratches across a 40-minute observation period for each treatment. Data are presented as mean \pm SEM values, $n = 6$ in each group. The asterisk (*) denotes significance vs vehicle control animals; * $P < 0.05$ (A, 2-way analysis of variance, followed by Bonferroni comparison post hoc test; B, unpaired student t -test).

unpaired Student *t*-test for immunoblotting results. Behavioural results are also represented as the total number of scratches across 40-minute observation period, and statistical analysis for these values was performed by unpaired Student *t* test. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Intradermal injection of CCI-779 reduces itch behaviour in mice

We investigated whether the inhibition of mTORC1 signaling pathway by i.d. injection of CCI-779 reduced the pruritic response evoked by i.d. histamine-dependent (compound 48/80) and histamine-independent (chloroquine, SLIGRL-NH2) pruritogens. Rapamycin and CCI-779 have been shown to inhibit mTORC1 activity within the spinal cord, dorsal roots, and cutaneous tissue and to inhibit mechanical hypersensitivity in rodent models of neuropathic pain.^{18,31} As indicated in **Figure 1**, i.d. injection of all 3 pruritogens induced scratching behaviour that lasted for ~40 minutes. Statistically significant inhibition of itch behaviour was observed for all 3 pruritogens and for the whole observation period when compared with vehicle control animals (**Fig. 1A**; compound 48/80: $F_{(1,77)} = 7.03$, $P = 0.009$; chloroquine: $F_{(1,80)} = 9.14$, $P = 0.003$; SLIGRL-NH2: $F_{(1,72)} = 30.14$, $P < 0.0001$). When CCI-779 was injected i.d. 6 hours before subsequent pruritogen

treatment, the total number of scratches induced by i.d. compound 48/80 and chloroquine (**Fig. 1B**) was significantly decreased by approximately $26.1 \pm 4\%$ ($t_{(10)} = 2.01$, $P = 0.04$) and $33.9 \pm 6.9\%$ ($t_{(10)} = 1.91$, $P = 0.04$), respectively. Additionally, in pilot studies, a 3-hour pretreatment period with i.d. CCI-779 yielded comparable results for chloroquine and compound 48/80 (data not shown). The strongest antipruritic action for CCI-779 was observed in SLIGRL-NH2-elicited scratching behaviour, with a reduction of $65.7 \pm 21.6\%$ (**Fig. 1A**; $t_{(9)} = 4.69$, $P = 0.0006$).

3.2. Systemic injection of CCI-779 reduces itch behaviour in mice

We also investigated whether systemic (i.p.) pretreatment with CCI-779 produced an antipruritic effect. As before, the dose of CCI-779 was as used previously²⁹ and was shown to inhibit mTORC1 activity and mechanical hypersensitivity in a mouse model of neuropathic pain and inflammatory hyperalgesia. CCI-779 was injected i.p. 6 hours before induction of scratching behaviour by compound 48/80, chloroquine, or SLIGRL-NH2 (**Fig. 2**). Pretreatment with CCI-779 significantly reduced the total number of scratches throughout the whole observation period for compound 48/80 and SLIGRL-NH2, with a reduction of $61.7 \pm 29.6\%$ and $44.3 \pm 10\%$, respectively (compound 48/80: $F_{(1,77)} = 39.19$, $P < 0.0001$; $t_{(10)} = 3.66$, $P = 0.004$; SLIGRL-NH2: $F_{(1,80)} = 32.15$,

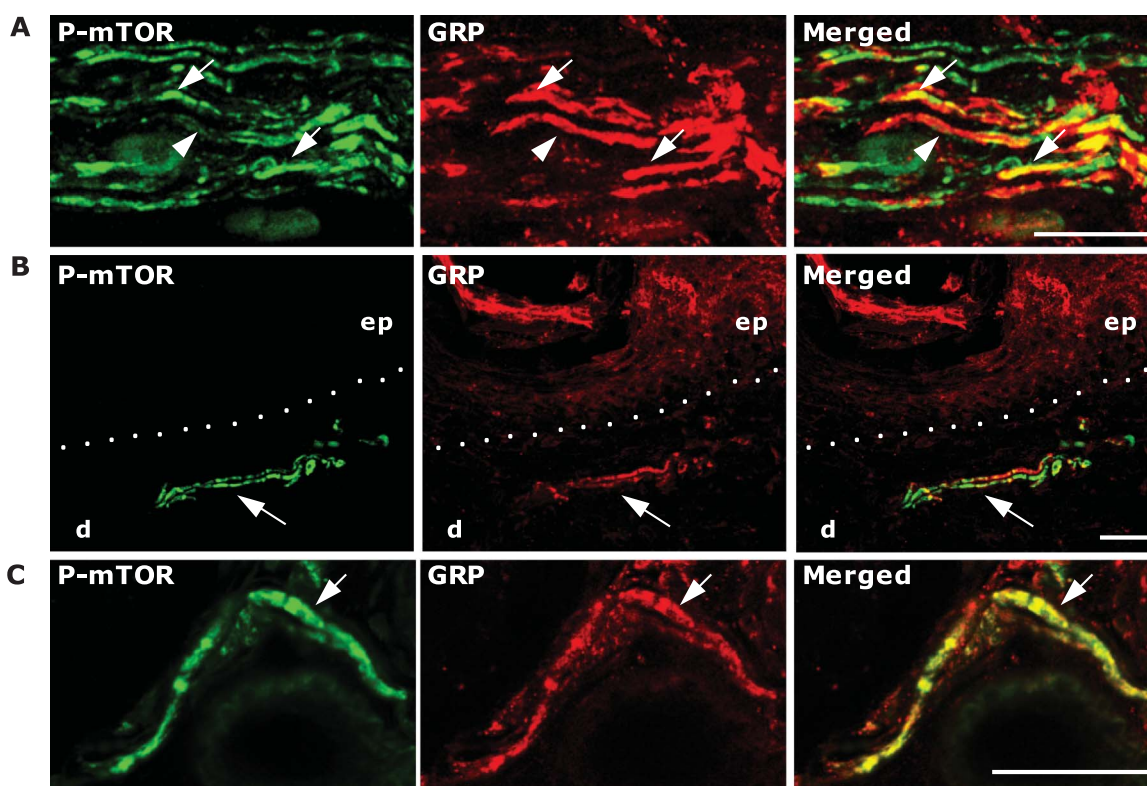


Figure 3. Phospho-mTOR (P-mTOR) immunoreactivity in the mouse skin was found within some gastrin-releasing peptide (GRP)-positive fibers. (A and B) Confocal images of 40- μm thick frozen sections of footpad of the glabrous skin cut perpendicular to the plantar skin surface of the mouse hind paw. (C) Confocal images of 40- μm thick frozen sections of hairy skin from the nape of the mouse neck cut perpendicular to the hairy skin surface. (A) Colocalization of P-mTOR (green) and GRP (red) in fibers contained within a bundle of axons within the dermis of the footpad. (B) Colocalization of P-mTOR (green) and GRP (red) in fibers within the dermis of the footpad. (C) Colocalization of P-mTOR (green) and GRP (red) in hairy skin. Arrows indicate double-labeled fibers. Arrowheads indicate GRP-positive fibers not double labeled. In (A-C) the single staining for each antibody and the merged image are shown from left to right and double staining appears in yellow. The dotted line in (B) indicates the border between the dermis and the epidermis of the mouse skin. ep: epidermis, d: dermis. (A-C) merged from 12 to 51 z-focal planes (12–35 μm depth). Scale bars, (A-C) = 25 μm .

$P < 0.0001$; $t_{(10)} = 3.36$, $P = 0.007$) (**Fig. 2A, B**). In chloroquine-elicited scratching behaviour, i.p. CCI-779 was only effective in the first 20 minutes of the itch response, with a $28.7 \pm 5.7\%$ reduction of scratching in this period (chloroquine: $F_{(1,37)} = 4.46$, $P = 0.04$; **Fig. 2A**).

3.3. P-mTOR is present in subsets of itch-sensitive fibers in the mouse skin

Immunohistochemical staining of skin sections of naive adult mouse hind paw showed that activated mTOR (phospho-mTOR, P-mTOR) was expressed in some subsets of itch sensitive afferents, identified from costaining with GRP, a putative marker for some itch-specific fibers (**Fig. 3**).^{41,42} P-mTOR labeling within GRP-positive axons was generally continuous and seen as either single fibers or more rarely within small bundles of axons within the dermis (**Fig. 3A**). We were able to trace single P-mTOR-double labeled axons for hundreds of micrometers within the dermis (**Fig. 3B**). Counts of single P-mTOR-positive fibers from the glabrous skin indicated that $<5\%$ of GRP-positive fibers contained P-mTOR ($n = 3$; 50–80 GRP fibers/animal monitored).

We also examined hairy skin sections from the base of the neck of naive adult mice, as this was the area used in mice to evaluate itch-related behaviour. Again, we found that P-mTOR was expressed in subsets of GRP-positive fibers but in contrast to the glabrous skin, and both P-mTOR and GRP positive fibers were less abundant in number and could be traced only for ~ 10 to $15 \mu\text{m}$ (**Fig. 3C**). There was also a slight decrease in the

number of double-stained fibers present, with $<3\%$ of GRP-positive fibers contained P-mTOR ($n = 3$; 30–50 GRP fibers per animal monitored).

3.4. Systemic injection of metformin reduces itch behaviour in mice

Metformin is a drug widely taken for type 2 diabetes and recently shown to inhibit mTORC1 signaling and to reduce mechanical hypersensitivity in a rodent model of neuropathic pain.²⁶ Here, systemic (i.p.) treatment with metformin injected 4 hours before pruritogens promoted a significant inhibition of SLIGRL-NH₂-elicited scratching behaviour with reduction in scratching of $40.2 \pm 11.1\%$ ($F_{(1,176)} = 21.64$, $P = 0 < 0.0001$; $t_{(22)} = 2.27$, $P = 0.02$; **Fig. 4**). Although the same metformin treatment had a significant inhibitory effect in the chloroquine-induced itch study with a reduction in scratching of $32.4 \pm 5.9\%$ ($F_{(1,72)} = 6.82$, $P = 0.01$), the total number of scratches in 40-minute period did not show a significant difference ($t_{(10)} = 1.34$, $P = 0.11$; **Fig. 4**). In addition, i.p. metformin 4 hours before pruritogens did not significantly reduce scratching behaviour caused by histamine-dependent compound 48/80 (**Fig. 4**). In contrast, when metformin was injected i.p. 24 hours before pruritogens, it significantly inhibited compound 48/80, chloroquine-, and SLIGRL-NH₂-elicited scratching behaviour, although the antipruritic effect for compound 48/80 was the weakest and observed only 10 to 20 minutes after induction of itch (**Fig. 5**). The reduction of itch was as follows: for compound 48/80 $12.5 \pm 11.2\%$ ($F_{(1,24)} = 9.43$,

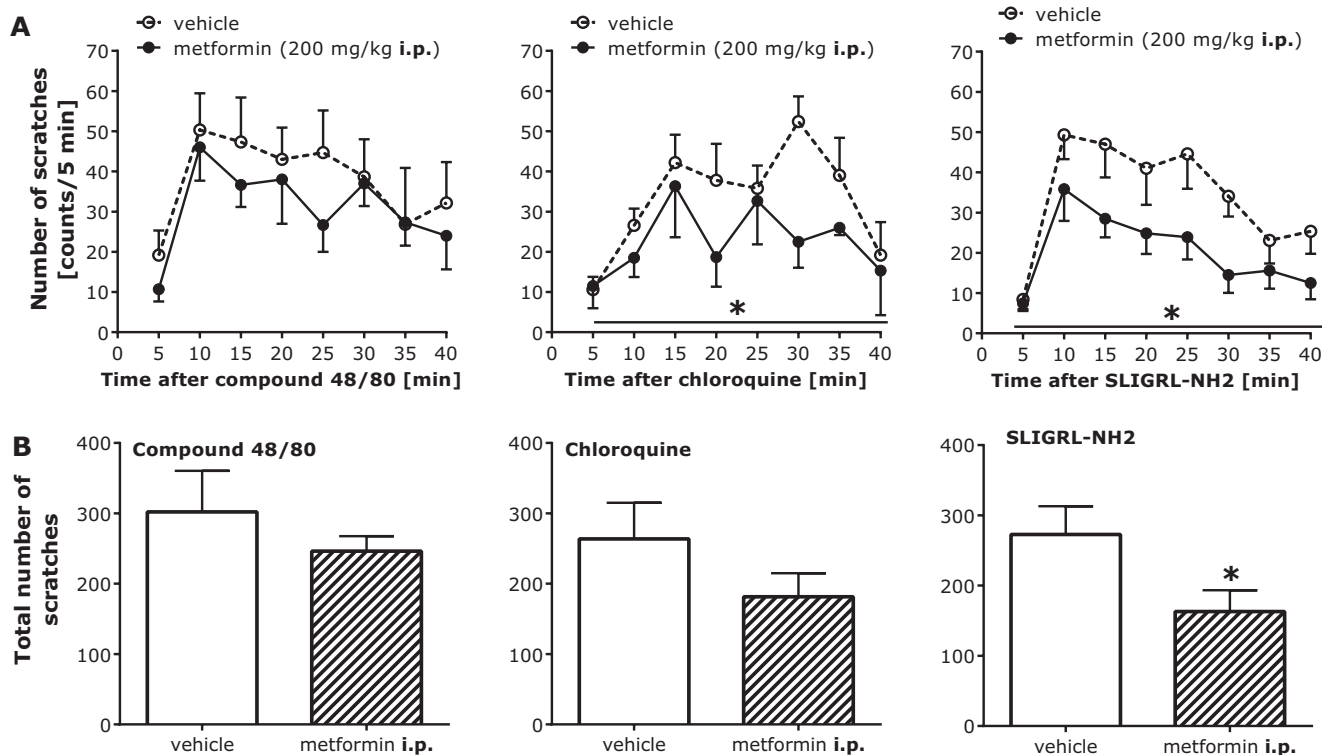


Figure 4. Intrapерitoneal (i.p.) administration of metformin 4 hours prior to pruritogens inhibited itch behaviour induced by nonhistaminergic pruritic agents in mice. (A) Time-course of i.p. metformin (200 mg/kg), or vehicle, in mice on the total number of scratches over a 40-minute period after i.d. injection into the nape of the mouse neck of compound 48/80, chloroquine, or SLIGRL-NH₂. Mice received an i.p. injection of metformin or vehicle 4 hours before the injection of pruritic agents. Itch behaviour was recorded and scratches were counted in 5-minute intervals for 40 minutes. (B) Bar graphs displaying a total number of scratches across 40-minute observation period for each treatment. Data are presented as mean \pm SEM values, $n = 6$ in each group. The asterisk (*) denotes significance vs vehicle control animals; * $P < 0.05$ (A, 2-way analysis of variance, followed by Bonferroni comparison post hoc test; B, unpaired student t -test).

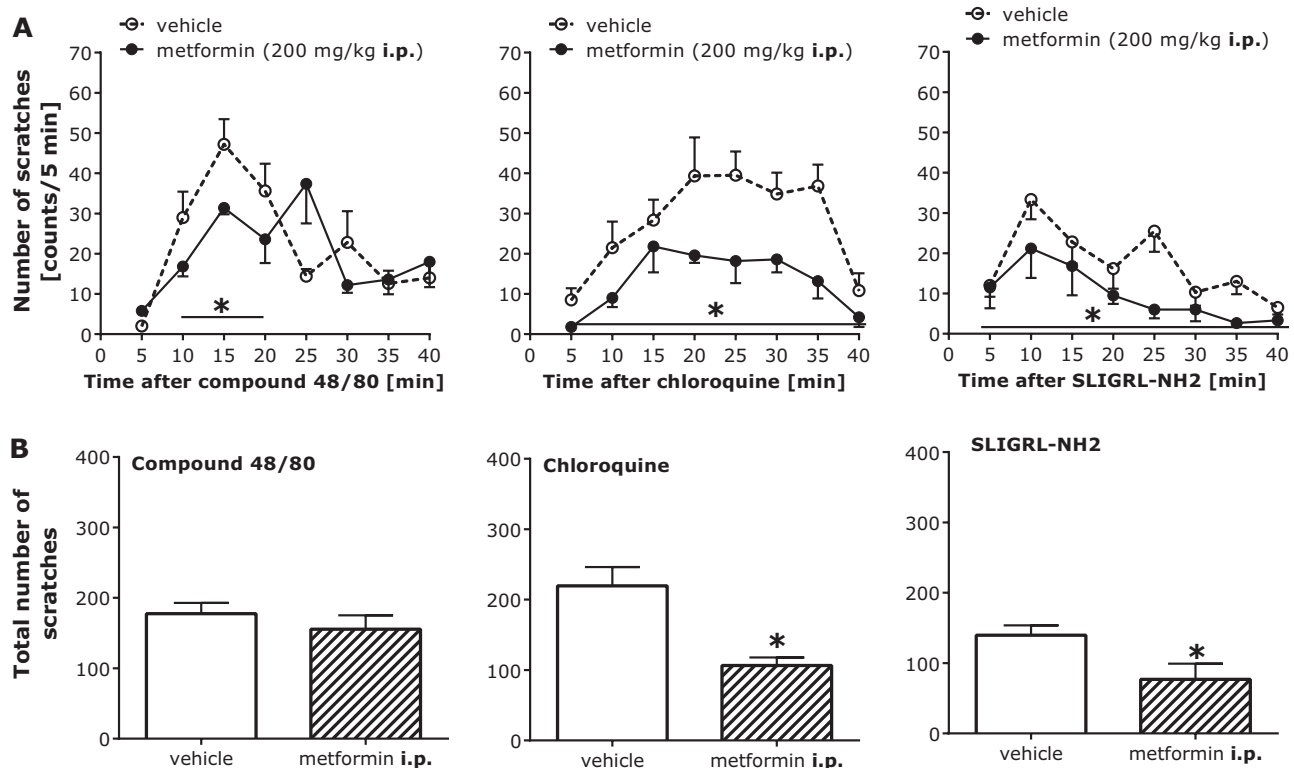


Figure 5. Intrapерitoneal (i.p.) administration of metformin 24 hours prior to pruritogens inhibited itch behaviour induced by histaminergic and nonhistaminergic pruritic agents in mice. (A) Time-course of i.p. metformin (200 mg/kg), or vehicle, in mice on the total number of scratches over a 40-minute observation period after i.d. injection into the nape of the mouse neck of compound 48/80, chloroquine, or SLIGRL-NH2. Mice received an i.p. injection of metformin or vehicle 24 hours before the injection of pruritic agents. Itch behaviour was recorded and scratches were counted in 5-minute intervals for 40 minutes. (B) Bar graphs displaying a total number of scratches across a 40-minute observation period for each treatment. Data are presented as mean \pm SEM values, $n = 6$ in each group. The asterisk (*) denotes significance vs vehicle control animals; * $P < 0.05$ (A, 2-way analysis of variance, followed by Bonferroni comparison post hoc test; B, unpaired student t -test).

$P = 0.005$; $t_{(8)} = 0.89$, $P = 0.4$), for chloroquine $51.6 \pm 5.2\%$ ($F_{(1,72)} = 29.53$, $P < 0.0001$; $t_{(9)} = 3.63$, $P = 0.003$), and for SLIGRL-NH2 $44.9 \pm 13.4\%$ ($F_{(1,80)} = 13.62$, $P = 0.0004$; $t_{(10)} = 2.38$, $P = 0.009$).

3.5. Systemic injection of metformin blocks mTORC1 activity in dorsal roots

In vitro metformin has been shown to inhibit mTORC1 signaling through activation of the AMPK pathway.^{6,26} We investigated whether metformin, at the dose effective in reducing itch-related behaviour, blocked the activity of mTORC1 in vivo in the spinal cord and dorsal roots by quantifying the phosphorylation level of direct downstream targets of mTORC1, S6 ribosomal protein, and 4E-BP1/2. Dorsal roots were used as they are made up almost exclusively of sensory fibers unlike the sciatic nerve that contains large number of motor axons. As indicated in **Fig. 6B**, systemic (i.p.) injection of metformin 4 hours before (at a dose previously evaluated²⁶) significantly reduced phosphorylation of 4E-BP1/2 in dorsal roots ($t_{(5)} = 3.9$, $P = 0.01$) but not in the spinal dorsal horn. When we analysed the activity of mTORC1 24 hours after i.p. injection of metformin, we observed a significant reduction of phosphorylation of mTORC1 and S6 ribosomal protein in dorsal roots ($t_{(10)} = 1.92$, $P = 0.04$ and $t_{(14)} = 2.13$, $P = 0.02$, respectively; **Fig. 7B**) but no changes in the spinal dorsal horn. However, a trend toward a reduction in 4E-BP1/2 did not reach significance. Taken together, these data confirm that mTORC1 activity was inhibited by i.p. metformin within sensory fibers in the periphery rather than in dorsal horn.

4. Discussion

There is a pressing need for new treatments for both acute and chronic pruritus, particularly those conditions that are insensitive to antihistamines.^{3,16,27,40} Here, we show that the rapamycin ester, CCI-779, attenuated both histaminergic and nonhistaminergic forms of itch, at a dose shown previously to inhibit the mTORC1 activity in spinal cord and dorsal roots.^{18,31} Furthermore, we showed that metformin treatment also specifically reduced nonhistamine-provoked itch and inhibited the mTORC1 (through measurement of P-4E-BP1/2) activity in dorsal roots. We therefore suggest that the reduction of the itch response seen after either CCI-779 or metformin treatment was caused by inhibition of mTORC1 within specific subsets of sensory primary afferent fibers. Our previous research has established that activated mTOR (P-mTOR) was mainly restricted to A-fibers, whereas there was little evidence for extensive expression within C-fibers.^{9,18,30,31,33} This correlated well with both electrophysiologic and behavioural deficits reported after local, intrathecal, or systemic (i.p.) treatment with the mTORC1 inhibitor rapamycin or rapalogue CCI-779.^{18,31} However, the mechanisms by which itch is signaled to the CNS are currently under intense scrutiny and considerable weight has been placed on signaling through specific C-fiber primary afferents.^{12,17,25,29,37} Nevertheless, the role of A-fibers in itch signaling is also established, if lacking in molecular detail.^{11,34} Thus, our current observations emphasize the role that A-fibers may play in itch signaling and the importance of the mTORC1 pathway in the regulation of some functions of these primary afferents.

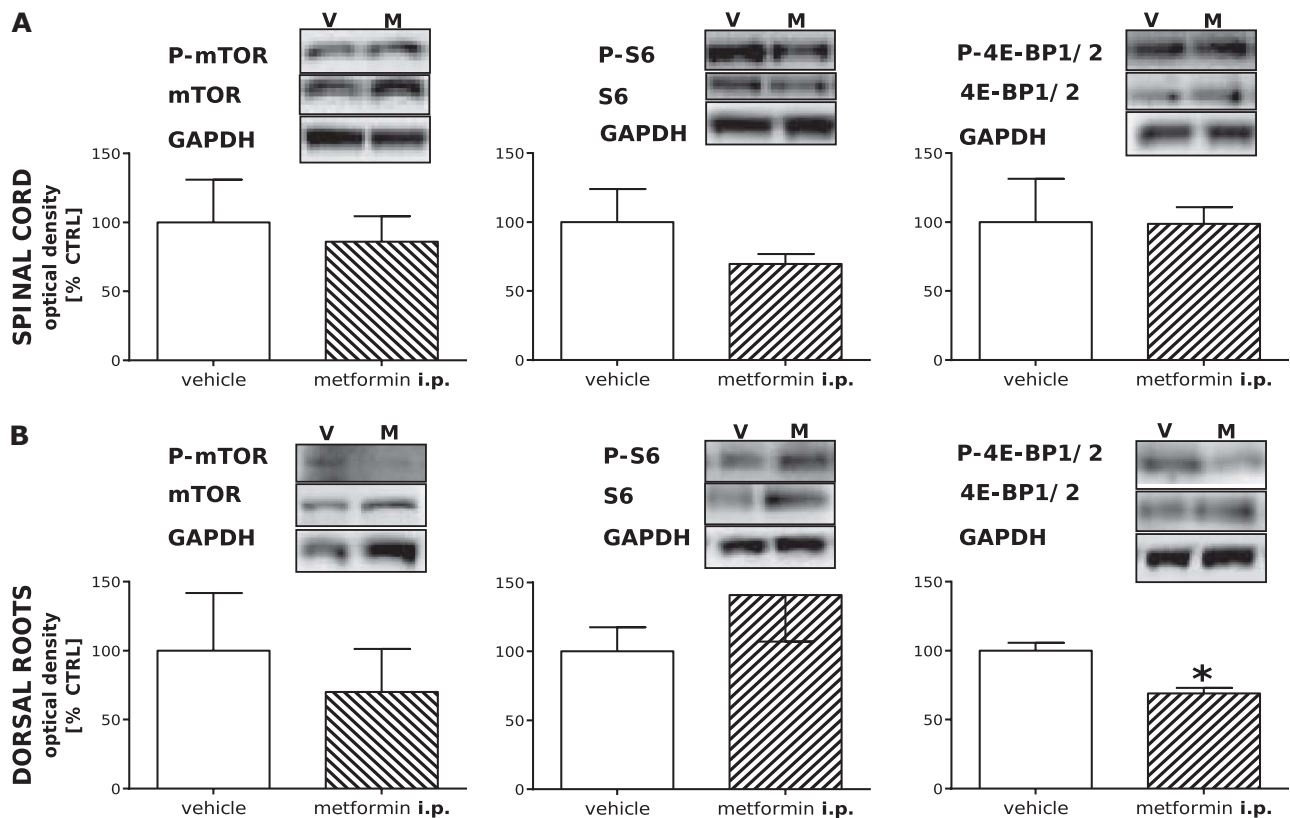


Figure 6. Intraperitoneal (i.p.) administration of metformin 4 hours prior to pruritogens decreased phosphorylation of the downstream target of mTORC1, 4E-BP1/2 in dorsal roots. (A and B) Effects of i.p. injection of metformin (M; 200 mg/kg), or vehicle (V), on the level phospho-mTOR (P-mTOR) and total mTOR, phospho-S6 ribosomal protein (P-S6), and total S6 protein, as well as phospho-4E-BP1/2 (P-4E-BP1/2) and total 4E-BP1/2 in the dorsal spinal cord (A) and dorsal roots (B). Mice received an i.p. injection of metformin or vehicle 4 hours before sacrifice. The intensity of the bands for each antibody was normalized with the intensity of its appropriate GAPDH signal. Each graph represents the phosphoprotein/total protein ratio, mean \pm SEM values, $n = 3$ to 6 per each experimental group. The asterisk (*) denotes significance vs vehicle controls; * $P < 0.05$ (unpaired Student t test). Representative immunoblots are shown above the appropriate graph bar.

4.1. Itch-specific primary afferent fibers

Evidence has been presented to show that GRP-containing primary afferent neurons and those expressing one of the Mas-related G protein-coupled receptor family members MrgprA3 were essential for the full expression of scratching after dermal injections of both the histamine-releasing compound 48/80 and the nonhistaminergic agents, chloroquine and SLIGRL-NH2 in mice.^{5,22,12} MrgprA3-expressing dorsal root ganglion (DRG) cells represent a very small population of largely C-fibers that project to the superficial dorsal horn where the majority synapse onto interneurons expressing the GRP receptor (GRPR).¹² In addition, recent research has suggested that the GRP-GRPR pathway is primarily engaged in transducing nonhistaminergic acute itch sensation and may play a relatively minor role in histaminergic itch.⁴⁶ Peripheral activation of dorsal horn neurons that express GRPR was also shown to be mediated through C-fibers and to involve glutamate release, although some A δ fiber signaling was also reported.²⁰ However, the location of GRP-immunoreactive neurons in neural circuits mediating itch remains controversial.^{39,43,45} Although Zhao et al.⁴⁵ reported resting levels of GRP in a small number of DRG neurons (~7%) confirming the original report of Sun et al.,⁴³ recent reports failed to find GRP in DRG cell bodies unless the peripheral nerve had been sectioned 3 days previously.³⁹ Here, we were able to detect for the first time a small number of GRP/P-mTOR colabelled primary afferent fibers in the mouse dermis. Given the controversy around the presence of GRP in DRGs, it may be that some GRP is locally transcribed

under the control of mTORC1 only in the terminal processes of a subpopulation of these GRP/P-mTOR-positive cutaneous afferents. If this was the case, it might also be suggested that in diseases with comorbid pruritus,¹⁶ circulating factors associated with the disease state may enhance the translation of peripheral GRP through activation of mTOR and thus facilitate itch. It should also be noted that the presence of GRPR was detected in the dermis of healthy human adults.⁴¹ Thus, together with our previous work on the characterization of P-mTOR-positive fibers, we would suggest that the small number of GRP and P-mTOR-positive fibers may well be the itch A δ fibers previously identified.³⁰ Therefore, inhibition of mTORC1 activity in these fibers potentially may contribute to the reduced itching seen after CCI-779 or metformin treatment.

4.2. Itch and A-fibers

Although labeled line theories of itch signaling have gained ground, a parallel series of investigations has uncovered complex interactions between primary afferents that were not predictable purely on the basis of a dedicated itch signaling pathway.^{35,28} For example, deletion of the vesicular glutamate transporter gene from primary afferents or ablation of a subset of inhibitory interneurons within the dorsal horn produces spontaneous itching and increased sensitivity to all types of pruritic stimuli. These results strongly imply that interactions between incoming primary afferents within the dorsal horn are necessary for the

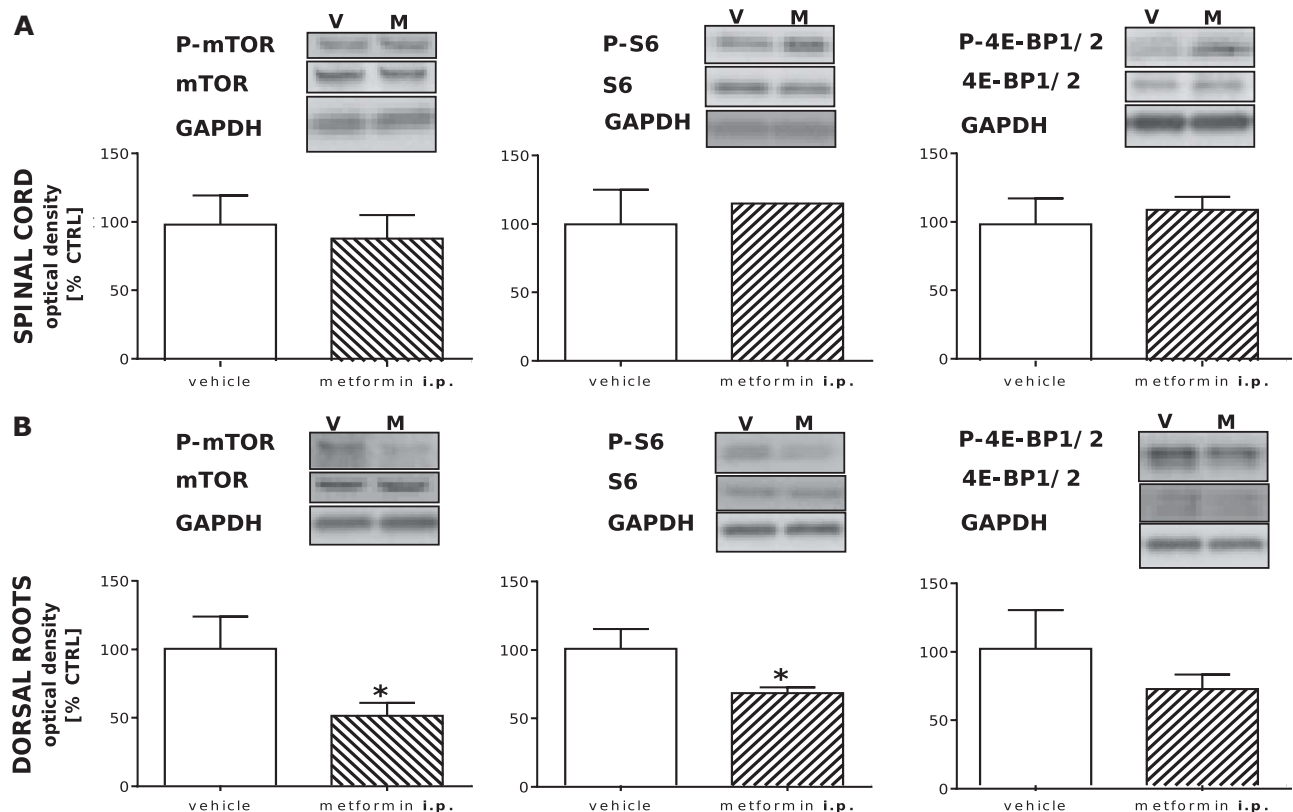


Figure 7. Intraperitoneal (i.p.) administration of metformin 24 hours prior to pruritogens decreased phosphorylation of mTORC1 and its downstream target, S6 ribosomal protein in dorsal roots. (A and B) Effects of i.p. injection of metformin (M; 200 mg/kg), or vehicle (V), on the level phospho-mTOR (P-mTOR) and total mTOR, phospho-S6 ribosomal protein (P-S6), and total S6 protein, as well as phospho-4E-BP1/2 (P-4E-BP1/2) and total 4E-BP1/2 in the dorsal spinal cord (A) and dorsal roots (B). Mice received an i.p. injection of metformin or vehicle 24 hours before sacrifice. The intensity of the bands for each antibody was normalized with the intensity of its appropriate GAPDH signal. Each graph represents the phosphoprotein/total protein ratio, mean \pm SEM values, $n = 3$ to 6 per each experimental group. The asterisk (*) denotes significance vs vehicle controls; * $P < 0.05$ (unpaired Student t -test). Representative immunoblots are shown above the appropriate graph bar.

experience of acute and chronic itch.^{21,23,36} Also, in addition to the well-known antagonistic interaction between pain and itch, similar patterns of peripheral and central sensitization have also been characterized. Noxious input to the spinal cord is known to provoke central sensitization reported behaviourally as allodynia and punctate mechanical hyperalgesia.^{4,11,14,16} Punctate mechanical hyperalgesia results in slightly painful pinprick stimulation being perceived as more painful in the secondary zone around a focus of inflammation or injury. Injury sets up central sensitization primarily through activation of C-fiber nociceptors, and this has been shown to amplify inputs from subsets of A-fibers recorded behaviourally as punctate mechanical hyperalgesia. These A-fibers also contain activated mTOR and local subcutaneous, intrathecal, or systemic treatment with mTORC1 inhibitors attenuated both experimentally induced punctate hyperalgesia and predictably neuropathic mechanical sensitivity in rodents.^{9,18,31} A strikingly similar pattern of central sensitization is observed in the itch pathway: local injection of a pruritic agent produces touch- or brush-evoked pruritus around the itching site.³ Hyperkinesis, itch becoming more intense in the area around pruritic stimulation, has been reported after histamine iontophoresis and provides compelling evidence for the existence of central sensitization for itch.^{2,4,27,36} It has also been shown that small myelinated fibers can contribute to both nonhistaminergic and histaminergic itch^{28,34} implying that there are parallel pathways signaling itch to the dorsal horn. Thus, itch-specific C-fibers trigger central sensitization, which amplifies itch-related

signals transmitted by A-fibers. Therefore, we suggest that inhibition of mTORC1 signaling pathways by CCI-779 may reduce activity in itch-sensitive A-fibers leaving C-fiber signaling intact. Indeed, it was found that itch sensation produced by cowhage (similarly to SLIGRL-NH₂-induced itch stimulation that is now confirmed to act at MrgprC11²²) was reduced during A-fiber block, and recent studies confirmed the contribution of A-fiber signaling to cowhage-induced itch and to histamine-mediated itch sensation.^{11,28,34}

4.3. Metformin as an antipruritic agent

We showed that systemic (i.p.) treatment with the biguanide drug metformin, widely given to treat type 2 diabetes, attenuated specifically scratching to nonhistaminergic (chloroquine and SLIGRL-NH₂) but less effectively histaminergic (compound 48/80) pruritic stimuli. We also showed that systemic treatment with metformin inhibited mTORC1-substrate eukaryotic initiation factor 4E-binding protein (4E-BP/2) and S6 ribosomal protein within the dorsal roots but not spinal cord. This was consistent with the observation that metformin and rapamycin or its analogs have been reported to have similar effects on mechanical hyperalgesia in neuropathic pain models.²⁶ As mentioned above, mTOR exists in 2 complexes: mTORC1, which is sensitive to the drug rapamycin, and mTORC2, which is not. The 2 best established substrates of mTORC1, p70-S6 kinase and 4E-BP1/2, control unique aspects of mRNA translation,²⁴ and

blocking mTORC1-regulated translation in A-fiber axons is thought to result in a decrease in primary afferent excitability.^{18,30} Rapamycin or rapalogues such as CCI-779 are allosteric inhibitors of mTORC1 through their binding to the immunophilin protein FKBP12 that binds specifically to mTORC1. The mechanisms behind the inhibition of protein translation by metformin are less well understood^{6,8} but involve upstream pathways of mTORC1 including activation of AMPK, as well as the AMPK effectors, LKB1, and tuberous sclerosis complex 2.^{6,8} The failure of metformin to inhibit histamine-sensitive itch was unexpected but may be accounted for by the fact that inhibition by metformin of mTORC1 (through 4E-BP1/2) activity is restricted to dorsal roots and not spinal cord (at this time point), whereas CCI-779 inhibition extends to the spinal cord.³¹ It is also likely that itch is signaled through overlapping subsets of primary afferent fibers,¹ which are in some cases insensitive to metformin inhibition. For example, it was recently reported that GRP antagonists would inhibit nonhistaminergic itch, but not that elicited by histamine, and that histamine responses at the H1 receptor on C-fibers were unique in requiring phospholipase C β as an intracellular mediator.^{1,13,42}

In conclusion, the data presented here provide, for the first time, evidence that the mTORC1 signaling pathway regulates both histamine-dependent and histamine-independent itch. Together with our previous studies on the role of mTORC1 in pain,^{9,18,30,31} this result may suggest that a subpopulation of P-mTOR-positive A-fibers may also be crucial for the full response to pruritic stimuli and therefore may underline the importance of the mTORC1 pathway in the regulation of homeostatic primary afferent functions such as pain and itch. Moreover, here we revealed a novel pharmacological action for metformin that may be therapeutically useful for the treatment of patients suffering from chronic pruritus not responsive to antihistamines.

Conflict of interest statement

The authors have no conflicts of interest to declare.

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